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(54) Title: NOVEL BRAIN EXPRESSED GENE AND PROTEIN ASSOCIATED WITH BIPOLAR DISORDER

(57) Abstract: We previously identified 18q21.33-q23 as a candidate region for bipolar (BP) disorder and constructed a yeast artificial chromosome (YAC) contig map. In a next step we isolated and analysed all CAG/CTG repeats from this region and excluded them from involvement in BP disorder. Here, in the process of identifying all CCG/CGG repeats from the region, we isolated three potential CpG islands, one of which is located 1.5 kb upstream of a predicted exon of 3639 bp. Further analysis showed this was part of a novel CpG-associated, brain-expressed gene, that we called NCAG1 (Novel CpG Associated Gene 1). Mutation analysis of this positional and functional candidate identified two single nucleotide polymorphisms, none of which were shown to be associated with the BP phenotype.

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## NOVEL BRAIN EXPRESSED GENE AND PROTEIN ASSOCIATED WITH BIPOLAR DISORDER

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### **FIELD OF THE INVENTION:**

The invention is broadly concerned with the determination of genetic factors associated with psychiatric health. More particularly, the present invention is directed to a human  
10 gene which is linked to a mood disorder or related disorder in affected individuals and their families. Specifically, the present invention is directed to a gene located on the eighteenth chromosome that is expressed in brain tissue and may be used as a diagnostic marker for bipolar disorder.

15

### **BACKGROUND OF THE INVENTION:**

#### **Pharmacogenetics background:**

20 Every individual is a product of the interaction of their genes and the environment. Pharmacogenetics is the study of how genetic differences influence the variability in patients responses to drugs. Through the use of pharmacogenetics, we will soon be able to profile variations between individuals DNA to predict responses to a particular medicine. Target validation that will predict a well-tolerated and effective medicine for  
25 a clinical indication in humans is a widely perceived problem; but the real challenge is target selection. A limited number of molecular target families have been identified, including receptors and enzymes, for which high throughput screening is currently possible. A good target is one against which many compounds can be screened rapidly to identify active molecules (hits). These hits can be developed into optimized  
30 molecules (leads), which have the properties of well-tolerated and effective medicines. Selection of targets that can be validated for a disease or clinical symptom is a major problem faced by the pharmaceutical industry. The best-validated targets are those that have already produced well-tolerated and effective medicines in humans (precedent targets). Many targets are chosen on the basis of scientific hypotheses and do not lead  
35 to effective medicines because the initial hypotheses are often subsequently disproved.

Two broad strategies are being used to identify genes and express their protein products for use as high-throughput targets. These approaches of genomics and genetics share technologies but represent distinct scientific tactics and investments. Discovery genomics uses the increasing number of databases of DNA sequence information to  
5 identify genes and families of genes for tractable or scrollable targets that are not known to be genetically related to disease.

The advantage of information on disease-susceptibility genes derived from patients is that, by definition, these genes are relevant to the patients' genetic contributions to the  
10 disease. However, most susceptibility genes will not be tractable targets or amenable to high-throughput screening methods to identify active compounds.

The differential metabolism related to the relevant gene variants can be studied in focused functional genomic and proteomic technologies to discover mechanisms of disease development or progression.

15 Critical enzymes of receptors associated with the altered metabolism can be used as targets. Gene-to-function-to-target strategies that focus on the role of the specific susceptibility gene variants on appropriate cellular metabolism become important.

Data mining of sequences from the Human Genome Project and similar programmes with powerful bioinformatic tools has made it possible to identify gene families by  
20 locating domains that possess similar sequences. Genes identified by these genomic strategies generally require some sort of functional validation or relationship to a disease process. Technologies such as differential gene expression, transgenic animal models, proteomics, in situ hybridization and immunohistochemistry are used to imply relationships between a gene and a disease.

25

The major distinction between the genomic and genetic approaches is target selection, which genetically defined genes and variant-specific targets already known to be involved in the disease process. The current vogue of discovery genomics for nonspecific, wholesale gene identification, with each gene in search of a relationship to  
30 a disease, creates great opportunities for development of medicines.

It is also critical to realize that the core problem for drug development is poor target selection. The screening use of unproven technologies to imply disease-related validation, and the huge investment necessary to progress each selected gene to proof

of a concept in humans, is based on an unproven and cavalier use of the word 'validation'. Each failure is very expensive in lost time and money. For example, differential gene expression (DGE) and proteomics are screening technologies that are widely used for target validation. They detect different levels and/or patterns of gene and protein expression in tissues, which may be used to imply a relationship to a disease affecting that tissue.

#### **Mood Disorder Background:**

Mood disorders or related disorders include but are not limited to the following disorders as defined in the Diagnostic and statistical Manual of Mental Disorders, version 4 (DSM-IV) taxonomy DSM-IV codes in parenthesis): mood disorders (296.XX,300.4,311,301.13,295.70) , schizophrenia and related disorders (295.XX,297.1,298.8,297.3,298.9), anxiety disorders (300.XX,309.81,308.3), adjustment disorders (309.XX) and personality disorders (codes 301.XX) .

The present invention is particularly directed to genetic factors associated with a family of mood disorders known as Bipolar (BP) spectrum disorders. Bipolar disorder (BP) is a severe psychiatric condition that is characterized by disturbances in mood, ranging from an extreme state of elation (mania) to a severe state of dysphoria (depression). Two types of bipolar illness have been described: type I BP illness (BPI) is characterized by major depressive episodes alternated with phases of mania, and type II BP illness (BPII) , characterized by major depressive episodes alternating with phases of hypomania. Relatives of BP probands have an increased risk for BP, unipolar disorder (patients only experiencing depressive episodes; UP), cyclothymia (minor depression and hypomania episodes; cy) as well as for schizoaffective disorders of the manic (SAm) and depressive (SAd) type. Based on these observations BP, cY, UP and SA are classified as BP spectrum disorders.

The involvement of genetic factors in the etiology of BP spectrum disorders was suggested by family, twin and adoption studies (Tsuang and Faraone (1990), the Genetics of Mood Disorders, Baltimore, The John Hopkins University Press) However, the exact pattern of transmission is unknown. In some studies, complex segregation analysis supports the existence of a single major locus for BP (Spence et al. (1995), Am J.Med. Genet (Neuropsych. Genet.) QQ pp 370-376). Other researchers propose a liability-threshold-model, in which the liability to develop the disorder results from the

additive combination of multiple genetic and environmental effects (McGuffin et al. (1994) , Affective Disorders; Seminars in Psychiatric Genetics Gaskell, London pp 110-127) .

Due to the complex mode of inheritance, parametric and non-parametric linkage  
5 strategies are applied in families in which BP disorder appears to be transmitted in a Mendelian fashion. Early linkage findings on chromosomes 11p15 (Egeland et al. (1987) , Nature ~ pp 783-787) and Xq27-q28 (Mendlewicz 'et al. (1987, the Lancet l pp 1230 -1232; Baron et al. (1987) Nature 12& pp 289-292) have been controversial and could initially not be replicated (Kelsoe et al. (1989) Nature ~ pp 238-243; Baron et al.  
10 (1993) Nature Genet ~ pp 49-55) .with the development of a human genetic map saturated with highly polymorphic markers and the continuous development of data analysis techniques, numerous new linkage searches were started. In several studies, evidence or suggestive evidence for linkage to particular regions on chromosomes 4, 12, 18, 21 and X was found (Black wood et al. (1996) Nature Genetics ~ pp 427-430,  
15 Craddock et al. (1994) Brit J. psychiatry ~ pp355-358, Berrettini et al. (1994), Proc Natl Acad Sci USA ~ pp 5918-5921, Straub et al. (1994) Nature Genetics ~ pp 291-296 and Pekkarinen et al. (1995) Genome Research 2 pp 105-115). In order to test the validity of the reported linkage results, these findings have to be replicated in other, independent studies.  
20 Recently, linkage of bipolar disorder to the pericentromeric region on chromosome 18 was reported (Berrettini et al. 1994). Also a ring chromosome 18 with break-points and deleted regions at 18pter-p11 and 18q23-qter was reported in three unrelated patients with BP illness or relates syndromes (Craddock et al. 1994). The chromosome 18p linkage was replicated by stine et al. (1995) Am J. Hum Genet 22 pp 1384-1394,  
25 who also reported suggestive evidence for a locus on 18q21.2-q21.32 in the same study.

Interestingly, Stine et al. observed a parent-of-origin effect: the evidence of linkage was the strongest in the paternal pedigrees, in which the proband's father or one of the proband's father's sibs is affected. Several studies described anticipation in families  
30 transmitting BP disorder(McInnis et al 1993, Nylander et al 1994) suggesting the involvement of trinucleotide repeat expansions (TREs), considering a number of diseases caused by an expansion of a CAG/CTG, a CCG/CGG or a GAA/TTC repeat show anticipation (reviewed by Margolis et al.(Margolis et al 1999)). Previous efforts

to find potentially expanded repeats have primarily focused on CAG/CTG repeats although the search for CCG/CGG repeats is increasing(Kleiderlein et al 1998, Mangel et al 1998, Eichhammer et al 1998, Kaushik et al 2000). Previously, we reported on a new method for the region specific isolation of triplet repeats: triplet repeat YAC  
5 fragmentation(Del Favero et al 1999). This proved to be a valid method for the isolation of CAG/CTG repeats and using this method, we excluded the involvement of CAG/CTG repeats from within 18q21.33-q23 in bipolar disorder(Goossens et al 2000). The present invention adapted the method for the region specific isolation of CCG/CGG repeats and applied it to the chromosome 18q21.33-q23 BP candidate  
10 region.

### **SUMMARY OF THE INVENTION:**

The present invention is directed to a novel gene and protein encoded by that gene.

The novel gene is located at an 8.9 cM chromosome region located between D18S68  
15 and D18S979 at 18q21.33-q23. A physical map was constructed using yeast artificial chromosomes (YACs)(Verheyen et al 1999).

The previously described method was adapted for the region specific isolation of CCG/CGG repeats and applied to the chromosome 18q21.33-q23 BP candidate region. Three potential CpG islands were isolated, one of which is located 1.5 kb upstream of  
20 a predicted exon of 3639 bp. Further analysis showed this was part of a novel CpG-associated, brain-expressed gene, herein called NCAG1 (Novel CpG Associated Gene 1). Mutation analysis of this positional and functional candidate identified two single nucleotide polymorphisms, which may be useful as a diagnostic marker for BP phenotype.

25

### **BRIEF DESCRIPTION OF THE DRAWING**

**Figure 1.** List of all human ESTs found by BLASTN alignment searches of dbEST. ESTs are named with their Genbank Acc Nos. I.M.A.G.E. Consortium [LLNL] cDNA  
30 Clones(Lennon et al 1996) are named with their RZPD clone ID.

**Figure 2:** Minimal YAC tiling path of the 18q21.33-q23 BP candidate region(Verheyen et al 1999). The YACs are represented by solid lines, the CCG/CGG

fragmentation products by dotted lines. YAC sizes, between brackets, are estimated by PFGE analysis. Solid circles indicate positive STS/STR hits. Shaded boxes highlight the CCG/CGG repeat and the three CpG islands isolated by YAC fragmentation.

- 5 **Figure 3:** Feature map of NCAG1. a) Predicted Features by bioinformatics. They encompass the CpG island as predicted by LCP(Huang 1994) and CPG(Larsen et al 1992), the ORF or exon as predicted by Grail(Uberbacher & Mural 1991) and Genscan(Burge & Karlin 1997), the transcription start site (TSS) as predicted by Proscan(Prestridge 1995)and the relevant polyadenylation signals as predicted by  
10 PolyAH(Salamov & Solovyev 1997). The numbers below the features indicate the scores as returned by Proscan and PolyAH. b) Alignment of EST hits. ESTs are named with their Genbank Acc Nos. c) Alignment of cDNA clones. I.M.A.G.E. Consortium [LLNL] cDNA Clones(Lennon et al 1996) are named with their RZPD clone ID. d)  
15 RT-PCR products. The grey bars represent the RT-PCR product, the thin black lines represent the sequences obtained on the nested PCRs.

#### **DETAILED DESCRIPTION OF THE INVENTION:**

The present invention is directed to a novel gene located at the 18q chromosomal  
20 candidate region of chromosome 18. More specifically, the gene is located at an 8.9 cM region located between D18S68 and D18S979 at 18q21.33-q23.

The gene is located at a chromosomal region associated with mood disorders such as bipolar spectrum disorders and may therefore be useful as a diagnostic marker for bipolar spectrum disorders. The region in question when removed from the totality of  
25 the human genome may also be used to locate, isolate and sequence other genes which influences psychiatric health and mood.

#### **Isolation and identification of Identification of novel gene:**

Standard procedures well-known to one skilled in the art were applied to the identified  
30 YAC clones and, where applicable, to the DNA from an individual afflicted with a mood disorder as defined herein, in the process of identifying and characterizing the relevant gene. For example, the inventors are able to make use of the previously identified apparent association between trinucleotide repeat expansions (TRE) within

the human genome and the phenomenon of anticipation in mood disorders (Lindblad et al. (1995), Neurobiology of Disease 2. pp 55-62 and O'Donovan et al. (1995), Nature Genetics 1Q pp 380-381) to screen for TRE's in the selected YAC clones in order to identify candidate genes in the region of interest on human chromosome 18. A variety of other known procedures can also be applied to the said YAC clones to identify the candidate gene as discussed below.

Accordingly, in a first aspect the present invention comprises the use of an 8.9 cM region of human chromosome 18q disposed between polymorphic markers D18S68 and D18S979 or a fragment thereof for identifying at least one human gene, including mutated and polymorphic variants thereof, which is associated with mood disorders or related disorders as defined above. As will be described below, the present inventors have identified this candidate region of chromosome 18q for such a gene, by analysis of co-segregation of bipolar disease in family MAD31 with 12 STR polymorphic markers previously located between D18S51 and D18S61 and subsequent LaD score analysis.

Particular YACs covering the candidate region which may be used in accordance with the present invention are 961.h-9, 942-c.3, 766-f-12, 731-c- 7, 907.e.1, 752-g-8 and 717-d-3, preferred ones being 961h-9, 766.f.12 and 907-e.1 since these have the minimum tiling path across the candidate region. suitable YAC clones for use are those having an artificial chromosome spanning the refined candidate region between D18S68 and D18S979.

There are a number of methods which can be applied to the candidate regions of chromosome 18q as defined above, whether or not present in a YAC, to identify a candidate gene or genes associated with mood disorders or related disorders. For example, as aforesaid, there is an apparent association between the extent of trinucleotide repeat expansions (TRE) in the human genome and the presence of mood disorders.

Accordingly, in a third aspect the present invention comprises a method of identifying at least one human gene, including mutated and polymorphic variants thereof, which is associated with a mood disorder or related disorder as defined herein which comprises detecting nucleotide triplet repeats in the region of human chromosome 18q disposed between polymorphic markers D18S68 and D18S979.

An alternative method of identifying said gene or genes comprises fragmenting a YAC clone comprising a portion of human chromosome 18q disposed between polymorphic markers D18S60 and D18S61, for example one or more of the seven aforementioned YAC clones, and detecting any nucleotide triplet repeats in said fragments, in particular  
5 repeats of CAG or CTG. Nucleic acid probes comprising at least 5 and preferably at least 10 CTG and/or CAG triplet repeats are a suitable means of detection when appropriately labelled. Trinucleotide repeats may also be determined using the known RED (repeat expansion detection) system (Shalling et al. (1993) , Nature Genetics ~ pp 135-139).

10 In a fourth embodiment the invention comprises a method of identifying at least one gene, including mutated and polymorphic variants thereof, which is associated with a mood disorder or related disorder and which is present in a YAC clone spanning the region of human chromosome 18q between polymorphic markers D18S60 and D18S61, the method comprising the step of detecting the expression  
15 product of a gene incorporating nucleotide triplet repeats by use of an antibody capable of recognizing a protein with an amino acid sequence comprising a string of at least 8, but preferably at least 12, continuous glutamine residues. Such a method may be implemented by sub-cloning YAC DNA, for example from the seven aforementioned YAC clones, into a human DNA expression library. A preferred means of detecting the  
20 relevant expression product is by use of a monoclonal antibody, in particular mAB1C2, the preparation and properties of which are described in International Patent.

Application Publication No WO 97/17445.

Further embodiments of the present invention relate to methods of identifying the relevant gene or genes which involve the sub-cloning of YAC DNA as defined above  
25 into vectors such as BAC (bacterial artificial chromosome) or PAC (P1 or phage artificial chromosome) or cosmid vectors such as exon-trap cosmid vectors. The starting point for such methods is the construction of a contig map of the region of human chromosome 18q between polymorphic markers D18S60 and D18S61. To this end the present inventors have sequenced the end regions of the fragment of human  
30 DNA in each of the seven aforementioned YAC clones and these sequences are disclosed herein. Following sub-cloning of YAC DNA into other vectors as described above, probes comprising these end sequences or portions thereof, in particular those sequences shown in Figures 1 to 11 herein, together with any known sequenced tagged

site (STS) in this region, as described in the YAC clone contig shown herein, as can be used to detect overlaps between said sub-clones and a contig map can be constructed. Also the known sequences in the current YAC contig can be used for the generation of contig map sub-clones.

- 5 One route by which a gene or genes which is associated with a mood disorder or associated disorder can be identified is by use of the known technique of exon trapping. This is an artificial RNA splicing assay, most often making use in current protocols of a specialized exon-trap cosmid vector. The vector contains an artificial mini-gene consisting of a segment of the SV40 genome containing an origin of replication and a  
10 powerful promoter sequence, two splicing-competent exons separated by an intron which contains a multiple cloning site and an SV40 polyadenylation site.

The YAC DNA is sub-cloned in the exon-trap vector and the recombinant DNA is transfected into a strain of mammalian cells. Transcription from the SV40 promoter results in an RNA transcript which normally splices to include the two exons of the  
15 minigene. If the cloned DNA itself contains a functional exon, it can be spliced to the exons present in the vector's minigene. Using reverse transcriptase a cDNA copy can be made and using specific PCR primers, splicing events involving exons of the insert DNA can be identified. Such a procedure can identify coding regions in the YAC DNA which can be compared to the equivalent regions of DNA from a person afflicted with  
20 a mood disorder or related disorder to identify the relevant gene.

Accordingly, in a fifth aspect the invention comprises a method of identifying at least one human gene, including mutated variants and polymorphisms thereof, which is associated with a mood disorder or related disorder which comprises the steps of:

- (1) transfecting mammalian cells with exon trap cosmid vectors prepared and mapped  
25 as described above;  
(2) culturing said mammalian cells in an appropriate medium;  
(3) isolating RNA transcripts expressed from the SV40 promoter;  
(4) preparing cDNA from said RNA transcripts;  
(5) identifying splicing events involving exons of the DNA sub-cloned into said exon  
30 trap cosmid vectors to elucidate positions of coding regions in said sub-cloned DNA;  
(6) detecting differences between said coding regions and equivalent regions in the DNA of an individual afflicted with said mood disorder or related disorder; and

(7) identifying said gene or mutated or polymorphic variant thereof which is associated with said mood disorder or related disorders.

As an alternative to exon trapping the YAC DNA may be sub-cloned into BAC, PAC, cosmid or other vectors and a contig map constructed as described above. There are a variety of known methods available by which the position of relevant genes on the sub-cloned DNA can be established as follows:

(a) cDNA selection or capture (also called direct selection and cDNA selection) : this method involves the forming of genomic DNA/cDNA heteroduplexes by hybridizing a cloned DNA (e.g. an insert of a YAC DNA), to a complex mixture of cDNAs, such as the inserts of all cDNA clones from a specific (e.g. brain) cDNA library. Related sequences will hybridize and can be enriched in subsequent steps using biotin-streptavidine capturing and PCR (or related techniques);

(b) hybridization to mRNA/cDNA: a genomic clone (e.g. the insert of a specific cosmid) can be hybridized to a Northern blot of mRNA from a panel of culture cell lines or against appropriate (e.g. brain) cDNA libraries. A positive signal can indicate the presence of a gene within the cloned fragment;

(c) CpG island identification: CpG or HTF islands are short (about 1 kb) hypomethylated GC-rich (> 60%) sequences which are often found at the 5' ends of genes. CpG islands often have restriction sites for several rare-cutter restriction enzymes. Clustering of rare-cutter restriction sites is indicative of a CpG island and therefore of a possible gene. CpG islands can be detected by hybridization of a DNA clone to Southern blots of genomic DNA digested with rare-cutting enzymes, or by island-rescue PCR (isolation of CpG islands from YACs by amplifying sequences between islands and neighbouring Alu-repeats) ;

(d) zoo-blotting: hybridizing a DNA clone (e.g. the insert of a specific cosmid) at reduced stringency against a Southern blot of genomic DNA samples from a variety of animal species. Detection of hybridization signals can suggest conserved sequences, indicating a possible gene. Accordingly, in a sixth aspect the invention comprises a method of identifying at least one human gene including mutated and polymorphic variants thereof which is associated with a mood disorder or related disorder which comprises the steps of:

(1) sub-cloning the YAC DNA as described above into a cosmid, BAC, PAC or other vector;

(2) using the nucleotide sequences shown in any one of Figures 1 to 11 or any other sequenced tagged site (STS) in this region as in the YAC clone contig described herein, or part thereof consisting of not less than 14 contiguous bases or the complement thereof, to detect overlaps amongst the sub-clones and construct a map thereof;

5 (3) identifying the position of genes within the sub-cloned DNA by one or more of CpG island identification, zoo-blotting, hybridization of the sub-cloned DNA to a cDNA library or a Northern blot of mRNA from a panel of culture cell lines;

(4) detecting differences between said genes and equivalent region of the DNA of an individual afflicted with a mood disorder or related disorder; and

10 (5) identifying said gene which is associated with said mood disorders or related disorders.

If the cloned YAC DNA is sequenced, computer analysis can be used to establish the presence of relevant genes. Techniques such as homology searching and exon prediction may be applied.

15 Once a candidate gene has been isolated in accordance with the methods of the invention more detailed comparisons may be made between the gene from a normal individual and one afflicted with a mood disorder such as a bipolar spectrum disorder. For example, there are two methods, described as "mutation testing", by which a mutation or polymorphism in a DNA sequence can be identified. In the first the DNA  
20 sample may be tested for the presence or absence of one specific mutation but this requires knowledge of what the mutation might be. In the second a sample of DNA is screened for any deviation from a standard (normal) DNA. This latter method is more useful for identifying candidate genes where a mutation is not identified in advance. In addition the following techniques may be further applied to a gene identified by the  
25 above-described methods to identify differences between genes from normal or healthy individuals and those afflicted with a mood disorder or related disorder:

(a) Southern blotting techniques: a clone is hybridized to nylon membranes containing genomic DNA digested with different restriction enzymes of patients and healthy individuals. Large differences between patients and healthy individuals can be  
30 visualized using a radioactive labelling protocol;

(b) heteroduplex mobility in polyacrylamide gels: this technique is based on the fact that the mobility of heteroduplexes in non-denaturing polyacrylamide gels is less than the mobility of homoduplexes. It is most effective for fragments under 200 bp;

(c) single-strand conformational polymorphism analysis (SSCP or SSCA) : single stranded DNA folds up to form complex structures that are stabilized by weak intramolecular bonds.

5 The electrophoretic mobilities of these structures on non-denaturing polyacrylamide gels depends on their chain lengths and on their conformation;

(d) chemical cleavage of mismatches (CCM) : a radiolabelled probe is hybridized to the test DNA, and mismatches detected by a series of chemical reactions that cleave one strand of the DNA at the site of the mismatch. This is a very sensitive method and can be applied to kilobase-length samples;

10 (e) enzymatic cleavage of mismatches: the assay is similar to CCM, but the cleavage is performed by certain bacteriophage enzymes.

(f) denaturing gradient gel electrophoresis: in this technique, DNA duplexes are forced to migrate through an electrophoretic gel in which there is a gradient of increasing amounts of a denaturant (chemical or temperature). Migration continues until the DNA  
15 duplexes reach a position on the gel wherein the strands melt and separate, after which the denatured DNA does not migrate much further. A single base pair difference between a normal and a mutant DNA duplex is sufficient to cause them to migrate to different positions in the gel;

(g) direct DNA sequencing.

20 It will be appreciated that with respect to the methods described herein, in the step of detecting differences between coding regions from the YAC and the DNA of an individual afflicted with a mood disorder or related disorder, the said individual may be anybody with the disorder and not necessary a member of family MAD31.

25 In accordance with further aspects the present invention provides an isolated human gene and variants thereof associated with a mood disorder or related disorder and which is obtainable by any of the above described methods, an isolated human protein encoded by said gene and a cDNA encoding said protein.

30 Once a gene has been identified a number of methods are available to determine the function of the encoded protein. These methods are described by Eisenberg et al (Nature vol. 15, June 2000) and is herein incorporated by reference. One method involves a computational method that reveals functional linkages from genome

sequences and is called the gene neighbor metho. If in several genomes the genes that encode two proteins are neighbors on the chromosome, the proteins tend to be functionally linked. This method can be powerful in uncovering functional linkages in prokaryotes, where operons are common, but also shows promise for analysing  
5 interacting proteins in eukaryotes.

### Examples:

#### Example 1

10

##### A: Triplet repeat isolation

CCG/CGG YAC fragmentation vectors were constructed by cloning blunted (CCG)<sub>10</sub>/(CGG)<sub>10</sub> adapters into the blunted SphI site of the previously described pDV1 basic vector(Del-Favero et al 1999). Sequencing determined that fragmentation vectors  
15 pDVCCG and pDVCGG have the adapter sequence in a 5'-(CCG)<sub>10</sub>-3' and a 5'-(CGG)<sub>10</sub>-3' orientation respectively.

Using these vectors, CCG/CGG repeats and flanking sequences were isolated by YAC fragmentation as described(Del-Favero et al 1999).

#### 20 B: Characterisation of Structure of the NCAG1 gene.

I.M.A.G.E. Consortium [LLNL] cDNA Clones(Lennon et al 1996)  
IMAGp998A136826Q2, IMAGp998A154307Q2, IMAGp998B194346Q2,  
IMAGp998D126826Q2, IMAGp998D193628Q2, IMAGp998F131866Q2,  
IMAGp998H201815Q2, IMAGp998K235214Q2, IMAGp998L153967Q2 and  
25 IMAGp998N06839Q2 were ordered at RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Heubnerweg 6, 14059 Berlin-Charlottenburg, Germany). Cultures starting from single colonies were grown and plasmids were prepared by the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). DNA sequencing was performed with the dideoxynucleotide sequencing method using a  
30 DNA sequencing kit (Perkin-Elmer, Foster, CA) and analysed by an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster, CA) or an ABI PRISM 3700 DNA Analyser (Perkin-Elmer, Foster, CA).

For the RT-PCR reactions, mRNA from *SHSY-5Y* cells was prepared using the  $\mu$ MACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). After  
35 DNaseI treatment (Promega, Madison, WI), the RT reaction was primed with

oligo(dT) primers and performed with Superscript Preamplification System for First Strand cDNA synthesis (GibcoBRL, N.V. Life Technologies, Merelbeke, Belgium). Fc-cDNA was used in long-range PCR reactions with TaKaRa LA Taq (Takara Shuzo Co., Otsu, Shiga, Japan). PCR products were reamplified with nested primers and  
5 sequenced as described above.

**C: Characterisation of the expression pattern of the NCAG1 gene.**

Genepool cDNA (Invitrogen, Carlsbad, CA) from brain, fetal brain, placenta, liver, testis and lung was used as a cDNA mapping panel. The Human Brain Multiple Tissue  
10 Northern (MTN) Blot IV (Clontech, Palo Alto, CA) was used for radioactive hybridisation in accompanying ExpressHyb solution according to the instructions of the manufacturer. A zooblot was prepared by digesting 10 µg genomic DNA to completion with HindIII, running it on a TAE 1% agarose gel and performing a Southern blot. A PCR product containing the ORF of the NCAG1 gene was radioactively labelled and  
15 hybridised at 65 °C.

**D: Mutation analysis of the NCAG1 gene.**

Overlapping PCR products of approximately 600 bp were generated and sequenced as described above. Both identified polymorphisms were detected by digesting the PCR  
20 product with HinfI and electrophoresing the fragments on precast ExcelGel gels on a Multiphor II electrophoresis system (Amersham Pharmacia Biotech AB, Uppsala, Sweden)

**E: CCG/CGG YAC fragmentation**

CCG/CGG YAC fragmentation was applied to YACs 961h9, 766f12 and 907e1(Goossens et al 2000). Size determination by Pulsed Field Gel Electrophoresis (PFGE) and Southern blot hybridisation resulted in 33 sets of equally sized fragmented YAC clones. Sequencing of 112 fragmented YAC ends identified seven (out of 33) sets  
30 of fragmented YACs with identical end sequences resulting from a specific homologous recombination. One set (CCG7) was the result of fragmentation in the (CGG)<sub>6</sub> repeat in the 5' UTR of the CAP2 gene (GenBank acc. No L40377). A second set (CCG6) contained a (CCG)<sub>2</sub> repeat and a third (CCG4) an imperfect CCCCCG repeat. The triplet repeat in the 5' UTR of the CAP2 gene was already shown not to be  
35 associated with BP disorder(Goossens et al 2000). The size of CCG4 was analyzed in

12 BP and 12 UP patients, but only one allele was detected. The size of CCG6 was not analyzed since it was too small to be polymorphic.

In depth analysis showed that three (CCG3, GenBank acc No ...; CCG4, GenBank acc No... and CCG6, GenBank acc No ...) of the seven sequences had high CG content  
5 (70-80 %) and high CpG content (15-20 CpGs in 200 bp) but no additional CCG/CGG repeats were found. Primer pairs for these potential CpG islands were used to determine their position on the YAC contig (Figure1). BLASTN analysis(Altschul et al 1990) resulted for both CCG4 and CCG6 in hits with sequences of RPCI-11 BACs. CCG4 gave a hit in a contig of 27150 bp of the working draft sequence of RPCI-11  
10 BAC 29O13 (GenBank acc No AC022662, GI: 7249117). CCG6 was part of the complete sequence of RPCI-11 BAC 793J2 (GenBank acc No AC009802).

**F: Identification and *in silico* characterisation of NCAG1 gene.**

To find genes possibly associated with the potential CpG islands CCG4 and CCG6,  
15 their surrounding BAC sequences were analysed using bioinformatic tools. Hence the 27150 bp contig of BAC 29O13 and the complete sequence of BAC 793J2 were sent for analysis to the Rummage High-Throughput Sequence Annotation Server (<http://gen100.imb-jena.de/rummage/index.html>).

First, LCP(Huang 1994) and CPG(Larsen et al 1992) recognized CpG islands  
20 containing CCG4 and CCG6 of 1.2 kb and 0.4 kb respectively, confirming their potential role as CpG islands.

In a next step, exon prediction programs Grail(Uberbacher & Mural 1991) and Genscan(Burge & Karlin 1997) both predicted the presence of a 3639 bp exon, 1.5 kb downstream of the 1.2 kb large CpG island containing CCG4. This predicted exon  
25 contains an open reading frame (ORF) which starts at an ATG start codon with an almost perfect Kozak sequence and ends with a TAA stop codon. Other predicted features are a transcription start site (TSS) at 2352 bp upstream of the ORF (score 76.6 by Proscan(Prestidge 1995)) and polyadenylation signals at 3032, 3247, 4364, 5338 and 8266 downstream of the ORF (respective scores of 4.79, 3.83, 4.94, 4.93 and 6.27  
30 by PolyAH(Salamov & Solovyev 1997)) (Figure2a).

BLASTN(Altschul et al 1990) alignment searches to sequences of dbEST revealed significant homology ( $\geq 97\%$ ) to 21 human ESTs (Table1, Figure2b). TBLASTX(Altschul et al 1997) searches of the Genbank non-redundant database (nr)

with the ORF showed extensive homology on protein level with SART-2 (Genbank  
Acc No NP\_037484), a squamous cell carcinoma antigen recognized by T-cells(Nakao  
et al 2000). Weaker homology was found with a series of sulfotransferases. Analysis of  
the 1212 long aminoacid sequence of the translated ORF by SMART (Simple Modular  
5 Architecture Research Tool, V3.1)(Schultz et al 2000) did not result in any known  
domains apart from a cleavable signal peptide at position 1-20 and two transmembrane  
segments at positions 771-791 and 800-820. Interpro reported no significant hits,  
although BLASTP(Altschul et al 1997) of the Prodom database showed homology  
between the NCAG1 gene and the chondroitin-6-sulfotransferase domain (Prodom Acc  
10 No PD042460)

**G: Characterisation of the structural organisation of the NCAG1 gene.**

Based on the BLASTN EST hits I.M.A.G.E. Consortium [LLNL] cDNA  
Clones(Lennon et al 1996) were ordered and sequenced. The sequences alligned with  
15 the genomic sequence in the presumed 5' UTR (untranslated region), the ORF and the  
presumed 3' UTR, indicating that these sequences are indeed transcribed (Figure2c).  
Alignment of the sequence of IMAGp998B194346Q2 with the genomic sequence  
showed that a 865 bp fragment was missing in the cDNA. A detailed analysis of the  
flanking sequences revealed the presence of consensus acceptor and donor splice sites,  
20 confirming that this fragment is probably an intron. Also clone IMAGp998D193628Q2  
missed a fragment of 1.9 kb when compared to the genomic sequence, but consensus  
splice sites were absent. Two clones, IMAGp998D193628Q2 and  
IMAGp998A136826Q2, terminated exactly at the predicted polyadenylation signal, 4.4  
kb downstream of the ORF. Sequences of clones IMAGp998A154307Q2,  
25 IMAGp998D126826Q2 and IMAGp998F131866Q2 did not align with the genomic  
sequence and were not analysed further.

Since cDNA clone sequencing did not result in a continuous sequence of the transcript,  
primers were designed and used for RT-PCR experiments. Sequencing of different  
overlapping RT-PCR products confirmed the presence of a transcript of at least 9 kb,  
30 containing the ORF of the predicted exon, linked to the presumed 5' and 3' sequences  
(Figure2d). The 5 prime intron of 865 bp was confirmed and the 3' UTR was extended  
till the predicted polyadenylation signal, 4.4 kb downstream of the ORF.

**H: Characterisation of the expression pattern of the NCAG1 gene.**

To investigate the expression profile of the NCAG1 gene, a long-range PCR spanning the ORF was optimised on genomic DNA and applied on a cDNA mapping panel. This showed that the fragment was present in cDNA from brain, fetal brain, placenta and  
5 liver but could not be detected in cDNA from testis and lung. More detailed information on the expression in the brain was obtained by Northern blot hybridisation showing expression of a  $\geq 9.5$  kb transcript in all investigated tissues (lung, placenta, small intestine, liver, kidney, skeletal muscle, heart, brain, uterus, trachea, thyroid, stomach, spinal cord, prostate, mammary gland, lymph node, brain (whole), bladder,  
10 adrenal gland, amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra, thalamus and total brain). Stringent Zooblot hybridisation experiments showed the presence of homologous sequences in the genomic DNA of other mammals like dog, pig, mouse, donkey, horse and sheep.

15

**I: Mutation analysis of the NCAG1 gene.**

Since this novel CpG-associated gene is brain-expressed and located in the chromosome 18q21.3-q23 BP candidate region, a mutation analysis of the ORF was performed on 3 patients and 1 escapee of the chromosome 18 linked family MAD31. In  
20 this way two single nucleotide polymorphisms were identified. The first is a C to T transition on position 2017 of the ORF, changing aminoacid (AA) 673 from proline to serine. This polymorphism was only found in the healthy control. The second polymorphism was found in all three patients. It was also a C to T transition, located at position 2824 and changing the 942 AA from proline to serine. Analysis of this  
25 polymorphism in family MAD31 showed that the T-allele was present on the disease haplotype.

Both polymorphisms were analysed in an association study on 92 BP patients and 92 age, sex and ethnicity matched controls by PCR-RFLP analysis. The P673S polymorphism turned out to be a frequent polymorphism with both alleles roughly  
30 equally present. The P942S polymorphism however was found to be a rare polymorphism, with the T allele only present in 3 BP patients and in 2 controls. Statistical analysis showed the control population was in Hardy-Weinberg equilibrium for both polymorphisms. No alleles, genotypes or haplotypes were found to be associated to BP disorder.

Since triplet repeat fragmentation was proven to be a valid method for the region specific isolation of triplet repeats(Goossens et al 2000), we applied it to the chromosome 18q21.33-q23 BP candidate region for the isolation of CCG/CGG repeats.

5 Therefore, we first had to construct a new set of fragmentation vectors, pDVCCG and pDVCGG. Fragmentation experiments with these vectors resulted in transformation and fragmentation efficiencies in the same range as obtained with the CAG/CTG fragmentation vectors pDVCAG and pDVCTG (data not shown). Application of CCG/CGG fragmentation to YAC 961h9 resulted in the isolation of the (CGG)<sub>6</sub> repeat  
10 in the 5' UTR of *CAP2*. This repeat is adjacent to the (CAG)<sub>6</sub> repeat previously reported(Goossens et al 2000). There, it was shown that this (CGG)<sub>6</sub>(CAG)<sub>6</sub> repeat is polymorphic but not expanded in BP cases nor associated with BP disorder. Taken together, the CCG/CGG YAC fragmentation data does not support CCG/CGG repeats as disease causing agents in chromosome 18q21.33-q23 linked BP disorder.

15 On the other hand, fragmentation experiments resulted in three sequences (CCG3, CCG4 and CCG6) with high CG (70 – 80 %) and CpG content but containing no CCG/CGG repeat. CpG islands are usually defined as regions of DNA of more than 200 bases that have a CG content above 50 % and a ratio of observed versus expected CpGs close to that statistically expected. Therefore, CCG3, CCG4 and CCG6 can be  
20 considered as potential CpG islands. Analysis of surrounding sequences of CCG4 and CCG6 with LCP(Huang 1994) and CPG(Larsen et al 1992) confirmed that the fragmentation occurred in both cases indeed in a CpG island. Since CpG islands are strongly associated with genes, more specifically housekeeping and widely expressed genes, these three sequences are likely to be located near this class of genes.

25 In the search for genes possibly associated with the isolated CpG islands, exon prediction programs Grail(Uberbacher & Mural 1991) and Genscan(Burge & Karlin 1997) both predicted the presence of a 3.6 kb exon downstream of the largest CpG island isolated. Two facts argued strongly against a false positive prediction. The first was that this two programs, based on different models, predicted exactly the same  
30 exon. The second was the mere presence in genomic DNA of this ORF continuing for 3.6 kb and starting with a Kozak consensus ATG. Additional evidence that this exon was indeed transcribed was found in the fact that a series of ESTs had very high homologies (97-100 %) with sequences in and surrounding the ORF. In a next step, this

- evidence was extended by sequencing of the cDNA clones from which the ESTs originated. The EST sequences were prolonged and corrected and the homologies increased to 99-100 %. The fact that the cDNA clones originated from different cDNA libraries (Table1) indicated that the gene was expressed in different tissues. RT-PCR and northern blot experiments resulted in the final confirmation that this ORF was widely expressed, a usual characteristic of a CpG-associated gene.
- cDNA clone sequencing resulted in complete sequence of seven human cDNA clones aligning with NCAG1. In two cases a piece of genomic DNA was missing in the cDNA sequence. Clone IMAGp998B194346Q2 lacked a 865 bp fragment (Figure2c). Since this fragment was flanked by splice donor and acceptor consensus sequences, and since the fragment was also missing in the RT-PCR products, enough evidence was gathered to call it an intron. Clone IMAGp998D193628Q2 also missed a 1.4 kb fragment compared to the genomic sequence. In this case no consensus splice sites were present. Moreover cDNA clones IMAGp998L153967Q2 and IMAGp998A136826Q2 contain sequences that are located in the missing fragment of IMAGp998D193628Q2 (Figure2c). This data together with the fact that EST AA442543 is located entirely in the missing fragment (Figure2b) and the presence of this fragment in the RT-PCR products (Figure2d) indicate that this fragment might rather be an artifact than an intron.
- EST-homologies and cDNA clone sequencing proved that a series of cDNA clones terminated at a predicted polyadenylation signal, 4.3 kb downstream of the ORF or 10.3 kb downstream of the predicted TSS. If the 5 prime intron of 865 bp is taken into account, the size of transcript will be 9.5 kb, which is the size of the transcript recognized in the Northern blot experiment.
- On protein level, a cleavable signal peptide and two transmembrane domains are predicted. If this is correct, both N-terminal and C-terminal sides will be at the same side of the membrane in which it is embedded. The strong homology with the SART-2 protein is significant, but it does not add more clues as to potential functions of the novel protein.
- The 2824T allele, present on the disease haplotype in the chromosome 18 linked family MAD31, is a very rare allele with a frequency of 0.03. Therefore statistical analysis in an association sample loses a lot of its strength, leaving the possibility that this allele confers an increased risk for BP disorder.

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5
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## CLAIMS

What is claimed is:

5

1. An isolated nucleic acid comprising the nucleotide sequence of SEQ ID N0: 1.

2. An isolated nucleic acid consisting essentially of the nucleotide sequence of SEQ ID N0: 1.

10

3. An isolated nucleic acid for comprising a nucleotide sequence that encodes the amino acid sequence of SEQ ID N0: 2.

4. An isolated nucleic acid comprising the nucleotide sequence of SEQ ID N0: 3.

15

5. An isolated nucleic acid consisting essentially of the nucleotide sequence of SEQ ID N0: 3.

20

6. An isolated nucleic acid consisting of the nucleotide sequence of SEQ ID N0: 1 or a contiguous fragment thereof wherein said isolated nucleic acid encodes a polypeptide having biological activity of bipolar disorder protein.

25

7. An isolated nucleic acid that hybridizes under high stringency conditions to a nucleic acid having a sequence complementary to the nucleotide sequence of SEQ ID N0: 1, wherein said isolated nucleic acid encodes a polypeptide having biological activity.

30

8. An isolated nucleic acid that encodes a polypeptide having the biological activity, said isolated nucleic acid consisting of a nucleotide sequence that is at least 90% identical to the nucleotide sequence of SEQ ID N0: 1.

35

9. An isolated nucleic acid consisting of the nucleotide sequence of SEQ ID N0: 3 or a contiguous fragment thereof wherein said isolated nucleic acid encodes a polypeptide having biological activity.

10. An isolated nucleic acid that hybridizes under high stringency conditions to a nucleic acid having a sequence complementary to the nucleotide sequence of SEQ ID N0: 3, wherein said isolated nucleic acid encodes a polypeptide having the biological activity.

5

11. An isolated nucleic acid that encodes a polypeptide having the biological activity,, said isolated nucleic acid consisting of a nucleotide sequence that is at least 90% identical to the nucleotide sequence of SEQ ID N0: 3.

10

12. Isolated and substantially purified protein encoded by the nucleic acid of Claim 6.

13. Isolated and substantially purified viral inhibitory protein 1 and 2 encoded by the nucleic acid of claim 9.

15

14. Isolated and substantially purified viral inhibitory protein having the amino acid sequence of SEQ ID N0: 2.

15. Isolated and substantially purified protein having an amino acid sequence that is at least 90% identical to the sequence of SEQ ID N0:2.

20

16. Isolated and substantially purified protein having an amino acid sequence that is at least 90% identical to the sequence of SEQ ID N0:4.

17. Isolated and substantially purified protein having an amino acid sequence that is at least 90% identical to the sequence of SEQ ID N0: 4.

25

18. A vector comprising the nucleic acid of claim 1.

19. A vector comprising the nucleic acid of claim 4.

30

20. A vector comprising the nucleic acid of claim 6 operable linked to an expression control sequence.

21. A host cell comprising the nucleic acid of claim 6.

35

22. A host cell comprising the vector of Claim 20.

23. A method of making protein 1 and 2 comprising:

- a) introducing the nucleic acid of claim 6 into a host cell;
- b) maintaining said host cell under conditions whereby said nucleic acid is expressed to protein;

5 c) recovering said protein.

24. A method of making protein comprising:

- a) introducing the nucleic acid of claim 9 into a host cell;
  - b) maintaining said host cell under conditions whereby said nucleic acid is expressed to
- 10 produce protein;
- c) recovering said protein.

25. A method of making protein comprising:

- a) introducing the nucleic acid of Claim 16 into a host cell;
- 15 b) maintaining said host cell under conditions whereby said nucleic acid is expressed to produce viral inhibitory protein;
- c) recovering said protein.

26. A composition comprising purified protein and a carrier.

20

27. The composition according to claim 26 which further comprises viral inhibitory protein 2.

## FIG 1

EST	cDNA library	I.M.A.G.E. clone
AA022684	Soares fetal heart NbHH19W	IMAGp998N06839Q2
AA022803	Soares fetal heart NbHH19W	IMAGp998N06839Q2
AA374532	HSC172 cells I	
AA421254	Soares ovary tumor NbHOT	IMAGp998H201815Q2
AA421255	Soares ovary tumor NbHOT	IMAGp998H201815Q2
AA442543	Soares total fetus Nb2HF8 9w	IMAGp998F131866Q2
AA858162	NCI CGAP Co8	IMAGp998D193628Q2
AI088531	Soares pregnant uterus NbHPU	IMAGp998A154307Q2
AI139422	Soares pregnant uterus NbHPU	IMAGp998B194346Q2
AI168185	Soares NSF F8 9W OT PA P S1	IMAGp998L153967Q2
AI401481	Soares NhHMPu S1	IMAGp998K235214Q2
AI732945	NCI CGAP Co8	IMAGp998D193628Q2
AI791264	NCI CGAP Co8	IMAGp998D193628Q2
AW015616	NCI CGAP Sub1	
AW139834	NCI CGAP Sub3	
AW450489	NCI CGAP Sub5	
BE139460	NCI CGAP Ut4	IMAGp998A136826Q2
BE139503	NCI CGAP Ut4	IMAGp998D126826Q2
C01922	Human adult	
C03813	Human heart	
R57132	Fetal heart	

FIG 2

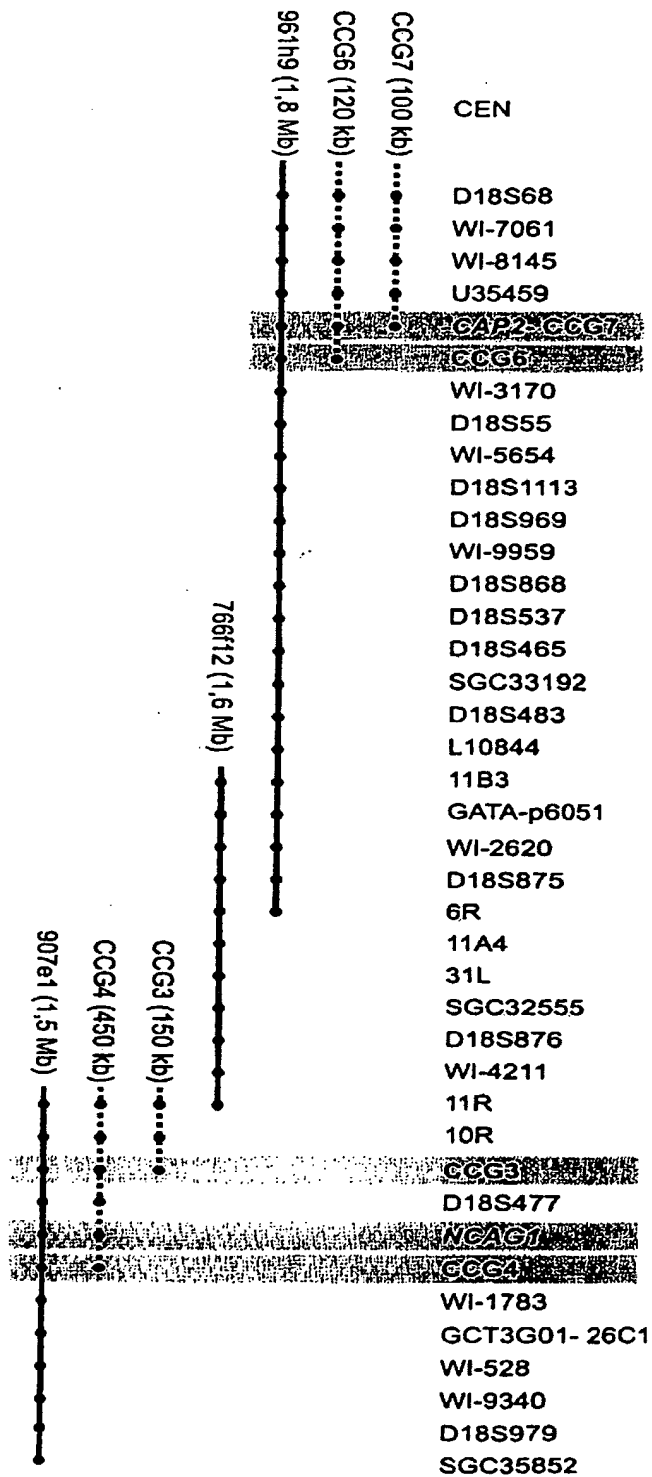
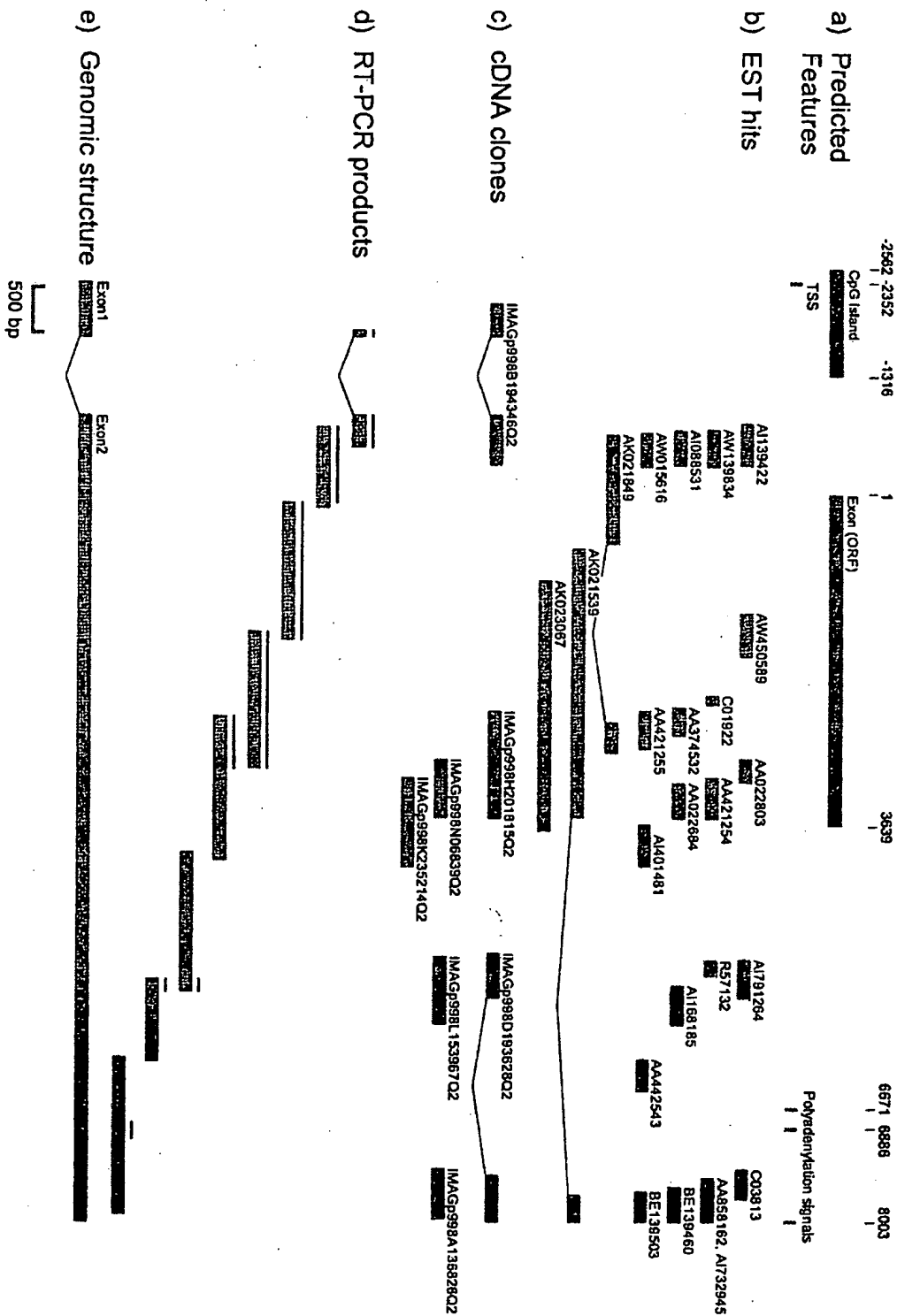


FIG 3



## SEQUENCE LISTING

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agcgtcgttg tctctgcctt tccctgaggg ccgccttca gccccgcctt caaccccgcc 180  
ccgtcctgcc tccgccccgc ccccgcttgc cggccccggt cgccgtctct caccctccc 240  
35 gggctgcgcg gccggagctg gcacagagga tcctcggccg cggcgacatc accgcctggg 300  
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         175                    180                    185                    190  
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10/22

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		165			170		175
5	Phe Ala Thr Ala Phe Asp Phe Leu Tyr Asn Leu Leu Asp Asn His Arg						
		180		185		190	
10	Arg Gln Lys Tyr Leu Glu Lys Ile Trp Val Ile Thr Glu Glu Met Tyr						
		195		200		205	
	Glu Tyr Ser Lys Val Arg Ser Trp Gly Lys Gln Leu Leu His Asn His						
		210		215		220	
15	Gln Ala Thr Asn Met Ile Ala Leu Leu Thr Gly Ala Leu Val Thr Gly						
			230		235		240
	Val Asp Lys Gly Ser Lys Ala Asn Ile Trp Lys Gln Ala Val Val Asp						
			245		250		255
20	Val Met Glu Lys Thr Met Phe Leu Leu Asn His Ile Val Asp Gly Ser						
		260		265		270	
	Leu Asp Glu Gly Val Ala Tyr Gly Ser Tyr Thr Ala Lys Ser Val Thr						
25		275		280		285	
	Gln Tyr Val Phe Leu Ala Gln Arg His Phe Asn Ile Asn Asn Leu Asp						
		290		295		300	
30	Asn Asn Trp Leu Lys Met His Phe Trp Phe Tyr Tyr Ala Thr Leu Leu						
			310		315		320
	Pro Gly Phe Gln Arg Thr Val Gly Ile Ala Asp Ser Asn Tyr Asn Trp						
			325		330		335
35	Phe Tyr Gly Pro Glu Ser Gln Leu Val Phe Leu Asp Lys Phe Ile Leu						
		340		345		350	
	Lys Asn Gly Ala Gly Asn Trp Leu Ala Gln Gln Ile Arg Lys His Arg						
40		355		360		365	
	Pro Lys Asp Gly Pro Met Val Pro Ser Thr Ala Gln Arg Trp Ser Thr						
		370		375		380	
45	Leu His Thr Glu Tyr Ile Trp Tyr Asp Pro Gln Leu Thr Pro Gln Pro						
			390		395		400
	Pro Ala Asp Tyr Gly Thr Ala Lys Ile His Thr Phe Pro Asn Trp Gly						
			405		410		415
50	Val Val Thr Tyr Gly Ala Gly Leu Pro Asn Thr Gln Thr Asn Thr Phe						
		420		425		430	
	Val Ser Phe Lys Ser Gly Lys Leu Gly Gly Arg Ala Val Tyr Asp Ile						
55		435		440		445	
	Val His Phe Gln Pro Tyr Ser Trp Ile Asp Gly Trp Arg Ser Phe Asn						
		450		455		460	
60	Pro Gly His Glu His Pro Asp Gln Asn Ser Phe Thr Phe Ala Pro Asn						
			470		475		480
	Gly Gln Val Phe Val Ser Glu Ala Leu Tyr Gly Pro Lys Leu Ser His						

11/22

					485						490					495
	Leu	Asn	Asn	Val	Leu	Val	Phe	Ala	Pro	Ser	Pro	Ser	Ser	Gln	Cys	Asn
				500					505					510		
5	Lys	Pro	Trp	Glu	Gly	Gln	Leu	Gly	Glu	Cys	Ala	Gln	Trp	Leu	Lys	Trp
			515					520					525			
10	Thr	Gly	Glu	Glu	Val	Gly	Asp	Ala	Ala	Gly	Glu	Ile	Ile	Thr	Ala	Ser
		530					535					540				
	Gln	His	Gly	Glu	Met	Val	Phe	Val	Ser	Gly	Glu	Ala	Val	Ser	Ala	Tyr
	545					550					555					560
15	Ser	Ser	Ala	Met	Arg	Leu	Lys	Ser	Val	Tyr	Arg	Ala	Leu	Leu	Leu	Leu
					565					570						575
	Asn	Ser	Gln	Thr	Leu	Leu	Val	Val	Asp	His	Ile	Glu	Arg	Gln	Glu	Asp
				580					585					590		
20	Ser	Pro	Ile	Asn	Ser	Val	Ser	Ala	Phe	Phe	His	Asn	Leu	Asp	Ile	Asp
			595					600					605			
	Phe	Lys	Tyr	Ile	Pro	Tyr	Lys	Phe	Met	Asn	Arg	Tyr	Asn	Gly	Ala	Met
25		610					615					620				
	Met	Asp	Val	Trp	Asp	Ala	His	Tyr	Lys	Met	Phe	Trp	Phe	Asp	His	His
	625					630					635					640
30	Gly	Asn	Ser	Pro	Met	Ala	Ser	Ile	Gln	Glu	Ala	Glu	Gln	Ala	Ala	Glu
					645					650						655
	Phe	Lys	Lys	Arg	Trp	Thr	Gln	Phe	Val	Asn	Val	Thr	Phe	Gln	Met	Glu
				660					665					670		
35	Pro	Thr	Ile	Thr	Arg	Ile	Ala	Tyr	Val	Phe	Tyr	Gly	Pro	Tyr	Ile	Asn
			675					680					685			
	Val	Ser	Ser	Cys	Arg	Phe	Ile	Asp	Ser	Ser	Asn	Pro	Gly	Leu	Gln	Ile
40		690					695					700				
	Ser	Leu	Asn	Val	Asn	Asn	Thr	Glu	His	Val	Val	Ser	Ile	Val	Thr	Asp
	705					710					715					720
45	Tyr	His	Asn	Leu	Lys	Thr	Arg	Phe	Asn	Tyr	Leu	Gly	Phe	Gly	Gly	Phe
					725					730						735
	Ala	Ser	Val	Ala	Asp	Gln	Gly	Gln	Ile	Thr	Arg	Phe	Gly	Leu	Gly	Thr
				740					745					750		
50	Gln	Ala	Ile	Val	Lys	Pro	Val	Arg	His	Asp	Arg	Ile	Ile	Phe	Pro	Phe
			755					760					765			
	Gly	Phe	Lys	Phe	Asn	Ile	Ala	Val	Gly	Leu	Ile	Leu	Cys	Ile	Ser	Leu
55		770					775					780				
	Val	Ile	Leu	Thr	Phe	Gln	Trp	Arg	Phe	Tyr	Leu	Ser	Phe	Arg	Lys	Leu
	785					790					795					800
60	Met	Arg	Trp	Ile	Leu	Ile	Leu	Val	Ile	Ala	Leu	Trp	Phe	Ile	Glu	Leu
					805					810					815	
	Leu	Asp	Val	Trp	Ser	Thr	Cys	Ser	Gln	Pro	Ile	Cys	Ala	Lys	Trp	Thr

12/22

	820	825	830
	Arg Thr Glu Ala Glu Gly Ser Lys Lys Ser Leu Ser Ser Glu Gly His		
	835	840	845
5	His Met Asp Leu Pro Asp Val Val Ile Thr Ser Leu Pro Gly Ser Gly		
	850	855	860
10	Ala Glu Ile Leu Lys Gln Leu Phe Phe Asn Ser Ser Asp Phe Leu Tyr		
	865	870	875
	Ile Arg Val Pro Thr Ala Tyr Ile Asp Ile Pro Glu Thr Glu Leu Glu		
	885	890	895
15	Ile Asp Ser Phe Val Asp Ala Cys Glu Trp Lys Val Ser Asp Ile Arg		
	900	905	910
	Ser Gly His Phe Arg Leu Leu Arg Gly Trp Leu Gln Ser Leu Val Gln		
	915	920	925
20	Asp Thr Lys Leu His Leu Gln Asn Ile His Leu His Glu Pro Asn Arg		
	930	935	940
25	Gly Lys Leu Ala Gln Tyr Phe Ala Met Asn Lys Asp Lys Lys Arg Lys		
	945	950	955
	Phe Lys Arg Arg Glu Ser Leu Pro Glu Gln Arg Ser Gln Met Lys Gly		
	965	970	975
30	Ala Phe Asp Arg Asp Ala Glu Tyr Ile Arg Ala Leu Arg Arg His Leu		
	980	985	990
	Val Tyr Tyr Pro Ser Ala Arg Pro Val Leu Ser Leu Ser Ser Gly Ser		
	995	1000	1005
35	Trp Thr Leu Lys Leu His Phe Phe Gln Glu Val Leu Gly Ala Ser Met		
	1010	1015	1020
40	Arg Ala Leu Tyr Ile Val Arg Asp Pro Arg Ala Trp Ile Tyr Ser Met		
	1025	1030	1035
	Leu Tyr Asn Ser Lys Pro Ser Leu Tyr Ser Leu Lys Asn Val Pro Glu		
	1045	1050	1055
45	His Leu Ala Lys Leu Phe Lys Ile Glu Gly Gly Lys Gly Lys Cys Asn		
	1060	1065	1070
	Leu Asn Ser Gly Tyr Ala Phe Glu Tyr Glu Pro Leu Arg Lys Glu Leu		
	1075	1080	1085
50	Ser Lys Ser Lys Ser Asn Ala Val Ser Leu Leu Ser His Leu Trp Leu		
	1090	1095	1100
55	Ala Asn Thr Ala Ala Ala Leu Arg Ile Asn Thr Asp Leu Leu Pro Thr		
	1105	1110	1115
	Ser Tyr Gln Leu Val Lys Phe Glu Asp Ile Val His Phe Pro Gln Lys		
	1125	1130	1135
60	Thr Thr Glu Arg Ile Phe Ala Phe Leu Gly Ile Pro Leu Ser Pro Ala		
	1140	1145	1150
	Ser Leu Asn Gln Ile Leu Phe Ala Thr Ser Thr Asn Leu Phe Tyr Leu		

1155                      1160                      1165  
 Pro Tyr Glu Gly Glu Ile Ser Pro Thr Asn Thr Asn Val Trp Lys Gln  
       1170                      1175                      1180  
 5    Asn Leu Pro Arg Asp Glu Ile Lys Leu Ile Glu Asn Ile Cys Trp Thr  
       185                      1190                      1195                      1200  
 10    Leu Met Asp Arg Leu Gly Tyr Pro Lys Phe Met Asp  
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       <211> 5092  
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 20    <220>  
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       <222> (501)..(4121)  
  
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 25    tctgagaatg acagtacttt atcatcttct tttggggaac atacagaaac ataccattta 60  
       tgtgtggttaa gttaatcact acagatgggt tcttgtgcta cgtggtcaaa tggcttcatt 120  
       tgaatttttg aatttttaaaa aattttttct ttttcacatg ttaattagat ttacacacag 180  
 30    ggagtaaattg ttggatttgt tgtattttct gactagacca ctgttttctg tgcattggag 240  
       acattggagg cattaatatt ccttgaaatt ttattttatt ggaagcaaac ctgtgccagg 300  
       gacacagaca tgctatataa tttcctaact tttcttgctt tgaataagct gaatgtcacc 360  
 35    tggatttcac agcctatgag gtatagtctg ttttttgttt ttgttttttt gctacatctt 420  
       taatatataa tttacaataa ccagatggga aacactgtgc ttaacacata tgcctaagga 480  
 40    aaagatcttc cccatggatc atg gcg ttt atg ttt aca gaa cat tta cta ttt 533  
                               Met Ala Phe Met Phe Thr Glu His Leu Leu Phe  
                               1                      5                      10  
  
 45    tta aca ttg atg atg tgt agt ttt tct act tgt gaa gaa tct gtg agc 581  
       Leu Thr Leu Met Met Cys Ser Phe Ser Thr Cys Glu Glu Ser Val Ser  
                               15                      20                      25  
  
       aat tat tct gaa tgg gca gtt ttc aca gac gat ata caa tgg ctt aag 629  
       Asn Tyr Ser Glu Trp Ala Val Phe Thr Asp Asp Ile Gln Trp Leu Lys  
                               30                      35                      40  
 50    tca cag aaa ata caa gat ttc aaa ctc aac cga aga ctt cat cca aat 677  
       Ser Gln Lys Ile Gln Asp Phe Lys Leu Asn Arg Arg Leu His Pro Asn  
                               45                      50                      55  
 55    tta tat ttt gat gct gga gat ata caa aca ttg aaa caa aag tct cgt 725  
       Leu Tyr Phe Asp Ala Gly Asp Ile Gln Thr Leu Lys Gln Lys Ser Arg  
                               60                      65                      70                      75  
 60    aca agc cat ttg cat att ttt aga gct atc aaa agt gca gtg aca att 773  
       Thr Ser His Leu His Ile Phe Arg Ala Ile Lys Ser Ala Val Thr Ile  
                               80                      85                      90

	atg ctg tcc aat cca tca tac tac cta cct cca ccc aag cat gct gag	821
	Met Leu Ser Asn Pro Ser Tyr Tyr Leu Pro Pro Pro Lys His Ala Glu	
	95 100 105	
5	ttt gct gcc aag tgg aat gaa att tat ggt aat aat ctt cct cct tta	869
	Phe Ala Ala Lys Trp Asn Glu Ile Tyr Gly Asn Asn Leu Pro Pro Leu	
	110 115 120	
10	gca ttg tat tgt tta tta tgc cca gaa gac aag gtt gcc ttt gaa ttt	917
	Ala Leu Tyr Cys Leu Leu Cys Pro Glu Asp Lys Val Ala Phe Glu Phe	
	125 130 135	
15	gtt atg gaa tac atg gat cgg atg gtt agc tac aaa gac tgg cta gtt	965
	Val Met Glu Tyr Met Asp Arg Met Val Ser Tyr Lys Asp Trp Leu Val	
	140 145 150 155	
20	gag aat gca cca ggg gat gag gtt cca gtt ggc cat tct tta aca ggt	1013
	Glu Asn Ala Pro Gly Asp Glu Val Pro Val Gly His Ser Leu Thr Gly	
	160 165 170	
25	ttt gcc act gcc ttt gac ttt tta tat aat cta tta ggt aat cag cgt	1061
	Phe Ala Thr Phe Asp Phe Leu Tyr Asn Leu Leu Gly Asn Gln Arg	
	175 180 185	
30	aaa caa aaa tac cta gaa aaa att tgg att gtt act gag gaa atg tat	1109
	Lys Gln Lys Tyr Leu Glu Lys Ile Trp Ile Val Thr Glu Glu Met Tyr	
	190 195 200	
35	gaa tat tcc aag att cga tca tgg ggc aaa caa ctt ctt cat aac cat	1157
	Glu Tyr Ser Lys Ile Arg Ser Trp Gly Lys Gln Leu Leu His Asn His	
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	Gln Ala Thr Asn Met Ile Ala Leu Leu Ile Gly Ala Leu Val Thr Gly	
	220 225 230 235	
45	gta gat aaa gga tct aaa gca aac ata tgg aaa caa gtt gtt gtt gat	1253
	Val Asp Lys Gly Ser Lys Ala Asn Ile Trp Lys Gln Val Val Val Asp	
	240 245 250	
50	gtg atg gaa aag act atg ttt ctc ttg aag cat att gta gat ggc tca	1301
	Val Met Glu Lys Thr Met Phe Leu Leu Lys His Ile Val Asp Gly Ser	
	255 260 265	
55	ttg gat gaa ggt gtg gcc tat gga agc tat acc tca aaa tca gtt aca	1349
	Leu Asp Glu Gly Val Ala Tyr Gly Ser Tyr Thr Ser Lys Ser Val Thr	
	270 275 280	
60	cag tat gtt ttt ttg gca caa cgc cat ttt aac atc aac aac ttt gat	1397
	Gln Tyr Val Phe Leu Ala Gln Arg His Phe Asn Ile Asn Asn Phe Asp	
	285 290 295	
65	aat aac tgg cta aaa atg cat ttt tgg ttt tat tat gct aca ctt ttg	1445
	Asn Asn Trp Leu Lys Met His Phe Trp Phe Tyr Tyr Ala Thr Leu Leu	
	300 305 310 315	
70	cca ggc tat caa aga act gta ggc ata gca gat tcc aat tat aat tgg	1493
	Pro Gly Tyr Gln Arg Thr Val Gly Ile Ala Asp Ser Asn Tyr Asn Trp	
	320 325 330	
75	ttt tat ggt cca gag agc cag cta gtt ttc ttg gat aag ttc att tta	1541
	Phe Tyr Gly Pro Glu Ser Gln Leu Val Phe Leu Asp Lys Phe Ile Leu	
	335 340 345	

5	cag aat gga gct gga aat tgg tta gct cag caa att aga aag cat cga	1589
	Gln Asn Gly Ala Gly Asn Trp Leu Ala Gln Gln Ile Arg Lys His Arg	
	350 355 360	
10	cct aag gat gga cca atg gtt cct tcc act gct cag cgg tgg agt act	1637
	Pro Lys Asp Gly Pro Met Val Pro Ser Thr Ala Gln Arg Trp Ser Thr	
	365 370 375	
15	ctt cat act gaa tac atc tgg tat gat cca aca ctc acc cca cag cct	1685
	Leu His Thr Glu Tyr Ile Trp Tyr Asp Pro Thr Leu Thr Pro Gln Pro	
	380 385 390 395	
20	cct gtt gat ttt ggc act gca aaa atg cac aca ttt cct aac tgg ggt	1733
	Pro Val Asp Phe Gly Thr Ala Lys Met His Thr Phe Pro Asn Trp Gly	
	400 405 410	
25	gtc gtg act tat ggg ggt ggg ctg cca aac acc cag acc aat acc ttt	1781
	Val Val Thr Tyr Gly Gly Gly Leu Pro Asn Thr Gln Thr Asn Thr Phe	
	415 420 425	
30	gtg tct ttt aaa tct ggg aaa ctg gga gga cga gct gtg tat gac ata	1829
	Val Ser Phe Lys Ser Gly Lys Leu Gly Gly Arg Ala Val Tyr Asp Ile	
	430 435 440	
35	gtt cac ttt cag cca tat tcc tgg att gat gga tgg aga agc ttt aac	1877
	Val His Phe Gln Pro Tyr Ser Trp Ile Asp Gly Trp Arg Ser Phe Asn	
	445 450 455	
40	cca gga cat gaa cat cca gat caa aat tca ttt act ttc gct cct aat	1925
	Pro Gly His Glu His Pro Asp Gln Asn Ser Phe Thr Phe Ala Pro Asn	
	460 465 470 475	
45	ggg cag gta ttc gtt tct gag gct ctt tat gga cca aaa ttg agc cac	1973
	Gly Gln Val Phe Val Ser Glu Ala Leu Tyr Gly Pro Lys Leu Ser His	
	480 485 490	
50	ctt aac aac gta ttg gtg ttt gcc cca tca cca tca agt caa tgt aat	2021
	Leu Asn Asn Val Leu Val Phe Ala Pro Ser Pro Ser Ser Gln Cys Asn	
	495 500 505	
55	cag ccc tgg gaa ggt caa ctg gga gaa tgt gca cag tgg ctc aag tgg	2069
	Gln Pro Trp Glu Gly Gln Leu Gly Glu Cys Ala Gln Trp Leu Lys Trp	
	510 515 520	
60	act ggg gaa gag gtt ggt gat gca gct ggg gaa gtt att act gct gct	2117
	Thr Gly Glu Glu Val Gly Asp Ala Ala Gly Glu Val Ile Thr Ala Ala	
	525 530 535	
65	caa cat ggt gat agg atg ttt gtg agt ggg gaa gca gtg tct gct tat	2165
	Gln His Gly Asp Arg Met Phe Val Ser Gly Glu Ala Val Ser Ala Tyr	
	540 545 550 555	
70	tct tct gcc atg aga ctg aaa agt gtc tat cgt gct tta ctt ctt tta	2213
	Ser Ser Ala Met Arg Leu Lys Ser Val Tyr Arg Ala Leu Leu Leu Leu	
	560 565 570	
75	aat tca caa act ctg ctt gtt gtc gat cat att gaa agg caa gaa act	2261
	Asn Ser Gln Thr Leu Leu Val Val Asp His Ile Glu Arg Gln Glu Thr	
	575 580 585	
80	tcc cca ata aat tct gtc agt gcc ttc ttt cat aat ttg gat att gat	2309
	Ser Pro Ile Asn Ser Val Ser Ala Phe Phe His Asn Leu Asp Ile Asp	

	590	595	600	
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10	atg gat gtg tgg gat gca cac tat aaa atg ttt tgg ttt gat cac cat Met Asp Val Trp Asp Ala His Tyr Lys Met Phe Trp Phe Asp His His 620 625 630 635	2405		
15	ggc aac agt cct gtg gct aat ata cag gaa gca gaa cag gct gct gaa Gly Asn Ser Pro Val Ala Asn Ile Gln Glu Ala Glu Gln Ala Glu 640 645 650	2453		
20	ttt aag aaa cgg tgg aca cag ttt gtt aat gtt aca ttt cat atg gaa Phe Lys Lys Arg Trp Thr Gln Phe Val Asn Val Thr Phe His Met Glu 655 660 665	2501		
25	tcc aca atc aca aga att gct tat gta ttt tat ggg cca tat gtc aat Ser Thr Ile Thr Arg Ile Ala Tyr Val Phe Tyr Gly Pro Tyr Val Asn 670 675 680	2549		
30	gtt tcc agc tgc aga ttt att gat agt tcc agt tct gga ctt cag att Val Ser Ser Cys Arg Phe Ile Asp Ser Ser Ser Ser Gly Leu Gln Ile 685 690 695	2597		
35	tct tta cat gtc aac agt act gaa cat agt gtg tct gtt gta act gac Ser Leu His Val Asn Ser Thr Glu His Ser Val Ser Val Val Thr Asp 700 705 710 715	2645		
40	tat caa aac ctt aaa agc aga ttc agt tac ctg gga ttt ggt ggt ttt Tyr Gln Asn Leu Lys Ser Arg Phe Ser Tyr Leu Gly Phe Gly Gly Phe 720 725 730	2693		
45	gcc agt gtg gct aat caa gga cag ata acc aga ttt ggt ttg ggt act Ala Ser Val Ala Asn Gln Gly Gln Ile Thr Arg Phe Gly Leu Gly Thr 735 740 745	2741		
50	caa gaa ata gta aac cct gta aga cat gat aaa gtt aat ttc ccc ttt Gln Glu Ile Val Asn Pro Val Arg His Asp Lys Val Asn Phe Pro Phe 750 755 760	2789		
55	ggg ttt aaa ttt aat ata gca gtt gga ttc att ttg tgt att agt ttg Gly Phe Lys Phe Asn Ile Ala Val Gly Phe Ile Leu Cys Ile Ser Leu 765 770 775	2837		
60	gtt att tta act ttt caa tgg cgg ttt tac ctt tcc ttt aga aag cta Val Ile Leu Thr Phe Gln Trp Arg Phe Tyr Leu Ser Phe Arg Lys Leu 780 785 790 795	2885		
65	atg cgc tgt gta tta ata ctt gtt att gcc ttg tgg ttt att gag ctt Met Arg Cys Val Leu Ile Leu Val Ile Ala Leu Trp Phe Ile Glu Leu 800 805 810	2933		
70	ctg gat gta tgg agt aca tgc act cag ccc atc tgt gca aaa tgg aca Leu Asp Val Trp Ser Thr Cys Thr Gln Pro Ile Cys Ala Lys Trp Thr 815 820 825	2981		
75	agg act gaa gct aag gca aat gag aag gtc atg att tct gaa ggg cat Arg Thr Glu Ala Lys Ala Asn Glu Lys Val Met Ile Ser Glu Gly His 830 835 840	3029		
80	cat gtg gat ctt cct aat gtt att att acc tca ctc cct ggt tca gga	3077		

	His	Val	Asp	Leu	Pro	Asn	Val	Ile	Ile	Thr	Ser	Leu	Pro	Gly	Ser	Gly	
	845						850					855					
5	gct	gaa	att	ctc	aaa	cag	ctt	ttt	ttc	aac	agc	agt	gat	ttt	ctc	tac	3125
	Ala	Glu	Ile	Leu	Lys	Gln	Leu	Phe	Phe	Asn	Ser	Ser	Asp	Phe	Leu	Tyr	
	860					865					870					875	
10	atc	aga	att	cct	aca	gcc	tac	atg	gat	atc	cct	gaa	act	gaa	ttt	gaa	3173
	Ile	Arg	Ile	Pro	Thr	Ala	Tyr	Met	Asp	Ile	Pro	Glu	Thr	Glu	Phe	Glu	
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15	att	gac	tca	ttt	gta	gat	gct	tgt	gag	tgg	aaa	gta	tca	gat	atc	cgc	3221
	Ile	Asp	Ser	Phe	Val	Asp	Ala	Cys	Glu	Trp	Lys	Val	Ser	Asp	Ile	Arg	
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	Ser	Gly	His	Phe	His	Leu	Leu	Arg	Gly	Trp	Leu	Gln	Ser	Leu	Val	Gln	
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	Asp	Thr	Lys	Leu	His	Leu	Gln	Asn	Ile	His	Leu	His	Glu	Thr	Ser	Arg	
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	Pro	Phe	Asp	Arg	Asp	Ala	Glu	Tyr	Ile	Arg	Ala	Leu	Arg	Arg	His	Leu	
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	Val	Tyr	Tyr	Pro	Ser	Ala	Arg	Pro	Val	Leu	Ser	Leu	Ser	Ser	Gly	Ser	
			990					995					1000				
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	Trp	Thr	Leu	Lys	Leu	His	Phe	Phe	Gln	Glu	Val	Leu	Gly	Thr	Ser	Met	
		1005					1010						1015				
55	cgg	gca	ttg	tac	ata	gta	aga	gac	cct	cga	gct	tgg	atc	tat	tca	gtg	3605
	Arg	Ala	Leu	Tyr	Ile	Val	Arg	Asp	Pro	Arg	Ala	Trp	Ile	Tyr	Ser	Val	
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5 aat tac cat ctg gtc aag ttt gaa gat att gtt cat ttt cct cag aag 3893  
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19/22

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15 Ala Val Phe Thr Asp Asp Ile Gln Trp Leu Lys Ser Gln Lys Ile Gln  
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Asp Phe Lys Leu Asn Arg Arg Leu His Pro Asn Leu Tyr Phe Asp Ala  
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Gly Asp Ile Gln Thr Leu Lys Gln Lys Ser Arg Thr Ser His Leu His  
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Ser Tyr Tyr Leu Pro Pro Pro Lys His Ala Glu Phe Ala Ala Lys Trp  
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Leu Cys Pro Glu Asp Lys Val Ala Phe Glu Phe Val Met Glu Tyr Met  
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Asp Phe Leu Tyr Asn Leu Leu Gly Asn Gln Arg Lys Gln Lys Tyr Leu  
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Arg Ser Trp Gly Lys Gln Leu Leu His Asn His Gln Ala Thr Asn Met  
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5	Ala	Asn	Ile	Gln	Glu	Ala	Glu	Gln	Ala	Ala	Glu	Phe	Lys	Lys	Arg	Trp	
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10	Ile	Ala	Tyr	Val	Phe	Tyr	Gly	Pro	Tyr	Val	Asn	Val	Ser	Ser	Cys	Arg	
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	Leu	Gln	Asn	Ile	His	Leu	His	Glu	Thr	Ser	Arg	Ser	Lys	Leu	Ala	Gln	
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 5 Ala Glu Tyr Ile Arg Ala Leu Arg Arg His Leu Val Tyr Tyr Pro Ser  
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 10 His Phe Phe Gln Glu Val Leu Gly Thr Ser Met Arg Ala Leu Tyr Ile  
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 45 Gly Tyr Pro Lys Phe Met Asp  
 1205

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